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We have identified 4 new CNS receptors for the circadian phase-resetting indoleamine neurotransmitter serotonin: 5-HT1F' 5-HT5A' 5-HT5B and 5-HT7. We found that the 5-HT5A receptor is located predominantly on astrocytes throughout the CNS, while the 5-HT7 receptor, which we have directly implicated in circadian phase-shifting, is located in the region of the suprachiasmatic nucleus that receives the primary retinal innervation. We have generated knock-out mice null for the 5-HT7 gene, but this mutation proved lethal when homozygous, hence we are unable to establish a genetic test its involvement in the mature nervous system. However, we have shown that this receptor is expressed in the thalamus and hypothalamus in the same neurons that are transcriptionally activated by the sleep-inducing lipid oleamide. We have characterized new neuropeptides, two (the hypocretins) expressed from a common precursor exclusively by a previously unrecognized nucleus within the hypothalamus, and another (cortistatin) expressed predominanyly by cortical and hippocampal interneurons that affects the onset of slow-wave sleep. To learn about the cellular mechanisms of entrainment, we have developed a new PCR-based methodology to identify mRNA molecules whose expression is specifically enriched in the SCN or whose accumulation there is stimulated by an entraining pulse of light, using RNA extracted from punched SCN tissue. The method has now been completely automated and has been coupled with a powerful bio-informatics network.

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I. SUMMARY

We have identified 4 new CNS receptors for the circadian phase-resetting indoleamine neurotransmitter serotonin: 5-HT_{1F}, 5-HT_{5A}, 5-HT_{5B} and 5-HT_7. We found that the 5-HT_{5A} receptor is located predominantly on astrocytes throughout the CNS, while the 5-HT_7 receptor, which we have directly implicated in circadian phase-shifting, is located in the region of the suprachiasmatic nucleus that receives the primary retinal innervation. We have generated knock-out mice null for the 5-HT_7 gene, but this mutation proved lethal when homozygous, hence we are unable to establish a genetic test its involvement in the mature nervous system. However, we have shown that this receptor is expressed in the thalamus and hypothalamus in the same neurons that are transcriptionally activated by the sleep-inducing lipid oleamide. We have characterized new neuropeptides, two (the hypocretins) expressed from a common precursor exclusively by a previously unrecognized nucleus within the hypothalamus, and another (cortistatin) expressed predominanyly by cortical and hippocampal interneurons that affects the onset of slow-wave sleep. To learn about the cellular mechanisms of entrainment, we have developed a new PCR-based methodology to identify mRNA molecules whose expression is specifically enriched in the SCN or whose accumulation there is stimulated by an entraining pulse of light, using RNA extracted from punched SCN tissue. The method has now been completely automated and has been coupled with a powerful bioinformatics network.

II. OBJECTIVES

The goal of the supported studies was to characterize new serotonin receptors and novel brain proteins so as to illuminate the molecular mechanisms that contribute to determination of circadian rhythms, with special emphasis on hypothalamus-specific mRNAs that regulate function of the suprachiasmic nucleus.

III. STATUS

a. Serotonin receptors

We have identified 4 new CNS receptors for the circadian phase-resetting indoleamine neurotransmitter serotonin: 5-HT_{1F}, 5-HT_{5A}, 5-HT_{5B} and 5-HT_7. We have generated antisera against two of these, 5-HT_{5A} and 5-HT_7, and used these in immunohistochemical mapping studies.

We found that the 5-HT_{5A} receptor is located predominantly on astrocytes throughout the CNS. Its timing of expression is concurrent with terminal astrocyte development and activation as judged by its coincidental detection with the glial fibrillary acidic protein (GFAP). Transfection of the receptor into glioma cells prevented the serotonin-induced increase in cAMP observed in untransfected cells and decreased the relative forskolin response by approximately 20%, suggesting that the 5-HT_{5A}

receptor couples negatively to adenylyl cyclase in astrocytes. Together these results indicate a neuron-to-astrocyte serotonergic signaling pathway mediating cAMP concentrations, which could provide a neuronally driven mechanism for regulating astrocyte physiology and neural inflammation.

We found that the 5-HT_7 receptor, which we have implicated in circadian phase-shifting, is located in the region of the suprachiasmatic nucleus that receives the primary retinal innervation, as well as in other areas of the hypothalamus and thalamus. To provide an explicit test of the involvement of this signalling circuit in circadian phase, we isolated the gene encoding the 5-HT_7 receptor from the mouse. We engineered an altered gene into which a reporter neomycin resistance gene had been introduced in a fashion that ablated the functionality of the receptor gene. The altered (null) gene was introduced into cultured embryonic stem cells by transfection and cells in which the altered gene had replaced the endogenous receptor gene were selected by growth in the neomycin analogue G418 and identified by a Southern blotting assay that discriminated heterologous from homologous insertion events. Several independent cell lines were injected into mouse blastocysts and founder mice carrying the null gene were identified. We bred a colony of heterozygous null gene carriers, however we found the null mutation to be lethal when bred to homozygosity. This is probably because the 5-HT_7 receptor is additionally expressed by nerves in the peripheral vascular system, hence the defect is incompatible with life in the developing embryos.

We investigated the effects of oleamide, an amidated lipid isolated from the cerebrospinal fluid of sleep-deprived cats, on serotonin-mediated responses. In rat P11 cells, which endogenously express the $5HT_2$ receptor, oleamide significantly potentiated serotonin-induced phosphoinosotide hydrolysis. In HeLa cells transfected with $5HT_7$, oleamide caused a concentration-independent increase in cAMP accumulation, but with lower efficacy than serotonin. This effect was not observed in untransfected cells. Clozapine did not affect the potentiation, but ketanserin inhibited the effect by 65%. In the presence of serotonin, oleamide had the opposite effect on cAMP, causing insurmountable antagonism of the concentration-effect curve to serotonin, but had no effect on cAMP levels elicited by isoproterenol or forskolin. These results indicate that oleamide can modulate serotonergic neurotransmission at different subtypes of 5HT receptors; and, additionally, that it acts at an apparent allosteric site on the $5HT_7$ receptor and elicits functional responses, either excitatory or inhibitory depending on the serotonin availability, via this site. This represents a novel mechanism of serotonin receptor regulation and may have pharmaceutical significance. In related studies, we have mapped the anatomical distribution of the degradative enzyme for oleamide within the brain.

To identify neuronal populations activated in vivo by

oleamide, we measured c-fos induction in the mouse brain in response to oleamide. Oleamide elicited dramatic increases in c-fos mRNA and protein in distinct brain regions, including cingulate and somatosensory cortical areas and numerous nuclei of the thalamus and hypothalamus. In the latter two areas, the majority of neurons induced for c-fos expressed the $5HT_7$ receptor, a target for oleamide in the in vitro studies. These data suggest that oleamide acts at $5HT_7$ receptors to elicit some of its physiological effects.

b. Peptides for feeding

We have continued to utilize subtractive hybridization to identify potential candidate genes for circadian regulation. One study identified the most prevalent mRNAs whose expression is enriched in the rat hypothalamus. Several of these were mapped at the cellular level by in situ hybridization. One was found to encode a 130-residue putative secretory protein with 4 sites for potential proteolytic maturation. Two of the putative products of proteolysis have 14 amino acid identities across 20 residues. This region includes a 7/7 match with a region of the gut hormone secretin, suggesting that the prepropeptide gives rise to two peptide products that are structurally related both to each other and to secretin. We isolated the entire mouse homologue. The mouse nucleotide sequence differs in 46 positions relative to the rat sequence and contains 13 additional nucleotides near its 3' end. Of these differences, 22 nucleotides differ within the protein coding region. Only 7 of these affect the encoded protein sequence. One amino acid difference is a neutral substitution in the secretion signal sequence. The remaining 6 differences are in the C-terminal region. One of these obliterates a potential proteolytic cleavage site. This observation and the nature of the other differences make it unlikely that 2 of the possible maturation products of the rat preproprotein are functional. However, the 2 peptides that are related both to each other and to secretin are absolutely preserved between species, providing strong support for the notion that these peptides have a function conserved during evolution.

The cells that express this mRNA are distributed in a bilaterally symmetrical pattern in a previously uncharted nucleus of the rat dorsal-lateral hypothalamus suggesting that the peptides function as intercellular messengers within the CNS. The rat mRNA is not expressed at high concentrations until 3 weeks after birth. In adults its concentration cycles during the day by greater than 2-fold with a maximum between CT13-16 (7-9pm), and that there is a 25% decrement after 6 hours of sleep deprivation.

The peptides are detected immunohistochemically in secretory vesicles at synapses of fibers that project to posterior hypothalamus and diverse targets in other brain regions. The peptides are excitatory when applied to cultured hypothalamic

neurons. Recent studies have identified the hypocretin peptides as ligands for two orphan receptors at which they stimulate feeding behavior. These peptides, recently discovered independently and called the orexins, accumulate during fasting.

c. **Sleep-Inducing peptide**

Using subtractive hybridization, we identified a clone of an mRNA encoding a novel rat neuropeptide, whose sequence shares 11/14 residues with somatostatin. We named the peptide cortistatin. Its precursor, preprocortistatin, is expressed postnatally in the rat brain in a subset of sparse GABAergic cortical and hippocampal neurons that partially overlap with those expressing somatostatin. A significant percentage of cortistatin-positive neurons is also positive for parvalbumin. In contrast, no co-localization was found between cortistatin and calretinin, cholecystokinin or VIP. During development, there is a transient increase in cortistatin-expressing cells in the second postnatal week in all cortical areas and in the dentate gyrus. A transient expression of preprocortistatin mRNA in the hilar region at P16 is paralleled by electrophysiological changes in dentate granule cells.

Synthetic cortistatin binds to all 5 cloned somatostatin receptors when they are expressed in transfected cells. In hippocampal slices, the peptide hyperpolarizes neurons by enhancing the M-current, a voltage-dependent potassium current. In contrast to somatostatin, administration of cortistatin into the rat brain ventricles specifically enhances slow wave sleep, apparently by antagonizing the effects of acetylcholine on cortical excitability. Cortistatin mRNA accumulates during sleep deprivation. A single amino acid difference with somatostatin accounts for the dramatic differences in the effects of the two peptides on physiology and behavior. Peptide analogues which preserve cortistatin-like functional activity bind sst3, suggesting that this receptor may mediate at least some of cortistatin's activity.

We identified cDNAs corresponding to mouse and human preprocortistatin. Analysis of the nucleotide and predicted amino acid sequences from rat and mouse reveals that the 14 C-terminal residues of preprocortistatin, which make up the sequence that is most similar to somatostatin, are conserved between species. Lack of conservation of other dibasic amino acid residues whose cleavage by prohormone convertases would give rise to additional peptides suggests that cortistatin-14 is the only active peptide derived from the precursor. As in the rat, mouse preprocortistatin mRNA is present in GABAergic interneurons in the cerebral cortex and hippocampus. The preprocortistatin gene maps to mouse chromosome 4, in a region showing conserved synteny with human 1p36. The human putative cortistatin peptide has an arginine for lysine substitution, compared to the rat and mouse products, and is N-terminally extended by 3 amino acids.

d. Peptides as pressures for voluntary but necessary behaviors

The studies on cortistatin and the hypocretins suggest a common mechanism of regulation for necessary, but voluntary, behaviors (sleep and feeding) by the presumably transcription-based accumulation of peptide transmitters that create pressures for the voluntary activities. Both cortistatin and the hypocretins accumulate as the physiological requirement for a particular behavior increases: for cortistatin, sleep; for the hypocretins, feeding. Both of these behaviors are necessary, but they are voluntary in that an animal has considerable flexibility as to when these needs must be satisfied.

Despite the restricted locations of the cell bodies expressing each of these peptides, each appears to be involved in more than a single system, and neither is the only signal for the behavior to which it has been most convincingly linked. Cortistatin is involved in sleep, but also appears to function in short term memory. Other substances have been implicated in sleep regulation: oleamide, for example. Similarly, the hypocretins are involved in feeding but, given their projections, probably several other processes including blood pressure and arousal. And, several additional neuropeptides have been implicated as promoters of food consumption: neuropeptide Y, galanin and melanin-concentrating hormone. Thus, to maintain flexibility in acceding to the multiplicity of demands imposed by internal physiology and the external natural and social environments, animals have evolved complex, overlapping neurohormonal signaling systems. One imagines that such overlapping systems allow both attention to individual demands and also integration of several demands, some of which may have conflicting solutions. We can expect that many additional signaling molecules remain to be found.

e. RNA identification methodologies

We have collaborated with a start-up biotechnology company, Digital Gene Technologies, to automate a method developed here that utilizes sequences near the 3′ ends of mRNA molecules to give each mRNA in an organism a unique identity, regardless of whether the mRNA has been discovered previously. The identity feature is used as part of a primer-binding site in PCR-based assays performed by robots on tissue extracts to determine the presence and relative concentration of nearly every mRNA in the extracts. We have developed informatics capabilities that display the comparisons of mRNA content of series of tissue sample in which each species is linked to corresponding genome database entries if they exist. The method is especially useful for discovery of mRNAs with anatomically restricted expression or that change during a physiological or pathological time course. We have now completed automatization of the method by robot and are begining a collaboration to identify all of the mRNAs that cycle at different circadian time points.

IV. PERSONNEL

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V. PUBLICATIONS

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press)

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VI. INTERACTIONS/TRANSITIONS

Program Committee, American Society for Neurochemistry, 1994-95

Editorial Boards: DNA and Cell Biology, Molecular Neurobiology Reviews, Journal of Neuroscience Research, Journal of Molecular Neuroscience, Journal of Neurogenetics, Advances in Neuroscience, Journal of Neurochemistry

Colloquium Chair and Lecture: American Society for Neurochemistry, Santa Monica, Ca (1995)

Chairman of Scientific Advisory Board and Director, Digital Gene Technologies: company established by The Scripps Research Institute to automate gene expression technology developed by principal investigator

Lectures: International Society for Developmental Neuroscience, San Diego (1994); Workshop on MouseMolecular Neurogenetics, Bar Harbor, Maine (1994); Behavioral Genetics Society, Richmond VA (1995); 5th International Workshop on Transcribed Sequences, Ile des Embiez, France (1995); Winter Conference on Brain Research, Snowmass CO (1996); 4th International Pituitary Conference, Coronado CA (1996); Workshop on Mouse Molecular Genetics, Bar Harbor, Maine (1996); 6th International Workshop on Transcribed Sequences, Edinburgh, UK (1996); Cambridge Healthtech Institute "Gene Functional Analysis" (1996); International Business Communications "Functional Genomics" (1996); American/International Societies for Neuroscience Joint Meeting (1997)

VIII. NEW DISCOVERIES, INVENTIONS, PATENT DISCLOSURES

Cortistatin: Neuropeptides, Compositions and Methods, US and foreign patents filed

Hypothalamus-specific polypeptides, U.S and foreign patent filed

IX. HONORS, AWARDS

Council, American Society for Neurochemistry
Society for Neuroscience Grass Traveling Lecturer